Chlamydiaceae and chlamydia-like organisms in free-living small mammals in Europe and Afghanistan

Stephan, S ; Guerra, D ; Pospischil, A ; Hilbe, Monika ; Weissenböck, H ; Novotny, L ; Greub, Gilbert ; Croxatto, A ; Teifke, J P ; Ulrich, R G ; Schlegel, M ; Ruhl, S ; Schotte, U ; Binder, A ; Sauer, S ; Borel, Nicole

Abstract: Few data are available on the occurrence of chlamydial infections in wild small mammals. Therefore, the present study aimed to investigate the significance of free-living small mammals as reservoirs or transmission hosts for chlamydiae. In total, 3,664 tissue samples originating from 911 animals were collected in Switzerland, Germany, Austria, the Czech Republic and Afghanistan. Samples included internal organs (n = 3,652) and feces (n = 12) from 679 rodents (order Rodentia) and 232 insectivores (order Eulipotyphla) and were tested by three different TaqMan real-time polymerase chain reactions (PCR) specific for members of the family Chlamydiaceae and selected Chlamydia-like organisms such as Parachlamydia spp. and Waddlia spp. Only one out of 911 (0.11%) animals exhibited a questionable positive result by Chlamydiaceae specific real-time PCR. Furthermore, five out of 911 animals (0.55%) were positive by specific real-time PCR for Parachlamydia spp. but could not be confirmed by Parachlamydia acanthamoebae secY qPCR. One out of 746 animals (0.13%) showed a positive result by real-time PCR for Waddlia chondrophila. This result was confirmed by Waddlia secY qPCR. This study represents the first detection of Chlamydia-like organisms in small wildlife in Switzerland. Considering previous negative results for Chlamydiaceae in wild ruminant species from Switzerland, these data suggest that wild small mammals are unlikely to be important carriers or transport hosts for Chamydiaceae and Chlamydia-like organisms.

DOI: https://doi.org/10.7589/2013-08-194

DOI: 10.7589/2013-08-194 *Journal of Wildlife Diseases*, 50(2), 2014, pp. 000–000

CHLAMYDIACEAE AND *CHLAMYDIA*-LIKE ORGANISMS IN FREE-LIVING SMALL MAMMALS IN EUROPE AND AFGHANISTAN

Sarah Stephan,[1] Diogo Guerra,[2] Andreas Pospischil,[1] Monika Hilbe,[1] Herbert Weissenböck,[3] Ladislav Novotný,[4] Gilbert Greub,[5] Antony Croxatto,[5] Jens Peter Teifke,[6] Rainer G. Ulrich,[6] Mathias Schlegel,[6] Silke Ruhl,[7] Ulrich Schotte,[7] Alfred Binder,[7] Sabine Sauer,[8] and Nicole Borel[1,9]

[1] Institute of Veterinary Pathology, University of Zurich, Vetsuisse Faculty Zurich, Winterthurerstrasse 268, 8057 Zurich, Switzerland
[2] Institute of Veterinary Parasitology, University of Zurich, Vetsuisse Faculty Zurich, Winterthurerstrasse 266a, 8057 Zurich, Switzerland
[3] Institute of Pathology and Forensic Veterinary Medicine, Department of Pathobiology, University of Veterinary Medicine Vienna, Veterinärplatz 1, 1210 Vienna, Austria
[4] Institute of Veterinary Pathology and Parasitology, Faculty of Veterinary Medicine, University of Veterinary and Pharmaceutical Sciences Brno, Palackého 1/3, 61242 Brno, Czech Republic
[5] Institute of Microbiology, University Hospital Center and University of Lausanne, Rue du Bugnon 48, 1011 Lausanne, Switzerland
[6] Friedrich-Löffler-Institut, Federal Research Institute for Animal Health, Südufer 10, 17493 Greifswald-Insel Riems, Germany
[7] Central Institute of the Bundeswehr Medical Service Kiel, Department of Veterinary Medicine, Koppenpahler Allee 120, 24119 Kronshagen, Schleswig-Holstein, Germany
[8] Central Institute of the Bundeswehr Medical Service Munich, Department of Veterinary Medicine, Ingolstädter Landstrasse 102, 85748 Garching, Freistaat Bayern, Germany
[9] Corresponding author (email: n.borel@access.uzh.ch)

ABSTRACT: Few data are available on the occurrence of chlamydial infections in wild small mammals. We investigated the significance of free-living small mammals as reservoirs or transmission hosts for microorganisms of the phylum/class *Chlamydiae*. We obtained 3,664 tissue samples from 911 animals in Switzerland, Germany, Austria, the Czech Republic, and Afghanistan. Samples included internal organs ($n=3,652$) and feces ($n=12$) from 679 rodents (order Rodentia) and 232 insectivores (order Soricomorpha) and were tested by three TaqMan® real-time PCRs specific for members of the family *Chlamydiaceae* and selected *Chlamydia*-like organisms such as *Parachlamydia* spp. and *Waddlia* spp. Only one of 911 (0.11%) animals exhibited a questionable positive result by *Chlamydiaceae*-specific real-time PCR. Five of 911 animals (0.55%) were positive by specific real-time PCR for *Parachlamydia* spp. but could not be confirmed by quantitative PCR targeting the *Parachlamydia acanthamoebae* secY gene (secY qPCR). One of 746 animals (0.13%) was positive by real-time PCR for *Waddlia chondrophila.* This result was confirmed by *Waddlia* secY qPCR. This is the first detection of *Chlamydia*-like organisms in small wildlife in Switzerland. Considering previous negative results for *Chlamydiaceae* in wild ruminant species from Switzerland, these data suggest that wild small mammals are unlikely to be important carriers or transport hosts for *Chamydiaceae* and *Chlamydia*-like organisms.

Key words: *Chlamydiaceae*, *Parachlamydia*, real-time PCR, *Waddlia*, wildlife.

INTRODUCTION

Members of the phylum/class *Chlamydiae* are obligate intracellular microorganisms that cause a variety of diseases in mammals and birds (Longbottom and Coulter, 2003). *Chlamydia muridarum*, the agent of the so-called mouse pneumonitis (MoPn), was previously classified as a biovar of *Chlamydia trachomatis.* It is composed of two strains, the Nigg strain (MoPn) and the SFPD strain (Zhang et al., 1993; Everett et al., 1999). Both have been isolated from laboratory mice and hamsters (Nigg, 1942; Stills et al., 1991). While *C. trachomatis* infects humans, closely related *C. muridarum* naturally occurs in members of the family Muridae, producing a subclinical respiratory infection in young laboratory albino Swiss mice (Nigg and Eaton, 1944). Infection by *C. muridarum* (MoPn) in laboratory mice was first observed by Dochez et al. (1937). Because all oculo-genital strains of *C.*

trachomatis infect mice when inoculated with highly infectious material (Storz and Page, 1971), laboratory mice became a widely used animal model for the investigation of human chlamydial infections (Laitinen et al., 1997). Strikingly, there is no report documenting isolation of *C. muridarum* from wild rodents. To our knowledge, no one has ever tried to isolate chlamydiae from wild insectivores such as shrews, hedgehogs, and moles.

Mouse models are widely used to provide insight into the pathogenesis of *Chlamydia abortus*, the agent of ovine enzootic abortion (OEA), because mice show the same clinical signs (abortion and pneumonia) as those observed in small ruminants (Caro et al., 2009). In Switzerland, prevalence of antibody to *C. abortus* in small ruminants was highest (43%) in the Canton of Grisons (Borel et al., 2004) where even wild ruminants are sporadic carriers of this abortigenic agent (e.g., Holzwarth et al., 2011a, b). As interactions between domestic or wild ruminants and free-living small mammals may occur on Alpine pastures, the role of the latter as reservoirs of *C. abortus* and other *Chlamydiaceae* should be considered.

In this survey we also searched for the *Chlamydia*-like organisms *Parachlamydia acanthamoebae* and *Waddlia chondrophila*. Both are considered to be important emerging pathogens in animals with zoonotic potential (Greub and Raoult, 2002) and they might be regarded as new abortigenic agents in Swiss and Scottish cows (e.g., Ruhl et al., 2009; Deuchande et al., 2010). Moreover, *P. acanthamoebae* has been associated with ocular lesions in naturally infected guinea pigs (*Cavia porcellus;* Lutz-Wohlgroth et al., 2006) and cats (*Felis catus*; Richter et al., 2010) and was recently shown to produce pneumonia in an experimental murine lung infection model (Casson et al., 2008b). *Parachlamydia* and *Waddlia* DNA have been detected in several ruminant wildlife species in Switzerland (Regenscheit et al., 2012) and even in environmental samples such as drinking and well water for cattle (*Bos primigenius*) (Wheelhouse et al., 2011; Codony et al., 2012). The potential of wild small mammals being a source of *Chlamydia*-like organisms has not been investigated.

Our aim was to elucidate the occurrence of *Chlamydiaceae*, focusing on *C. muridarum* and *C. abortus* and selected *Chlamydia*-like organisms such as *P. acanthamoebae* and *W. chondrophila*, in free-living small mammals of different geographic regions in the Old World such as Switzerland, Germany, Austria, the Czech Republic, and Afghanistan.

MATERIALS AND METHODS

In total, 3,652 tissue samples and 12 fecal samples from 911 wild small mammals of 20 species were available from Switzerland ($n=490$), Afghanistan ($n=379$), Germany ($n=29$), Austria ($n=8$), and the Czech Republic ($n=5$; Table 1 and Supplementary Material). Animals ($n=490$) from Switzerland originated from five Swiss cantons: Grisons ($n=277$), Lucerne ($n=201$), Aargau ($n=8$), Fribourg ($n=3$), and Zurich ($n=1$). Samples for Grisons were available from two recent studies investigating shrews as a reservoir host of Borna disease virus (Hilbe et al., 2006; Puorger et al., 2010). Animals originating from Lucerne were available as part of a collaborative project with the Institute of Veterinary Parasitology, University of Zurich and were obtained in August and November 2012. Samples from other Cantons were provided by private individuals. All animals were trapped in live or kill traps, and for each captured animal species sex, age, and origin were recorded. Small mammals originating from Afghanistan ($n=379$) were trapped in military camps of the International Security Assistance Force (ISAF) in Mazar-e-Sharif ($n=302$), Kunduz ($n=51$), and Fayzabad ($n=26$) between November 2010 and March 2011 and necropsied according to standard protocol (Schlegel et al., 2012a). We used DNA preparations of the kidney and the liver. Additionally, small mammals from Germany ($n=29$) and Austria ($n=8$) were collected by the Department of Pathobiology, University of Veterinary Medicine, Vienna. Mice from the Czech Republic ($n=5$) originating from the South Moravia Region were provided by the Faculty of Veterinary Medicine, Brno. For animals from Germany, Austria, and the

TABLE 1. Details of 911 small animals tested for *Chlamydiaceae* and *Chlamydia*-like organisms.

Order	Family	Genus	Species	*n*	Origin[a]
Rodentia	Muridae	*Apodemus*	Wood mouse (*Apodemus sylvaticus*)	33	CH
			Alpine field mouse (*Apodemus alpicola*)	3	CH
			Yellow-necked mouse (*Apodemus flavicollis*)	19	CH
		Mus	*Mus* sp.	12	CH
			House mouse (*Mus musculus*)	332	AFG
		Rattus	*Rattus* sp.	1	AFG
	Cricetidae	*Arvicola*	Fossorial water vole (*Arvicola scherman*)	126	CH
		Cricetulus	Gray dwarf hamster (*Cricetulus migratorius*)	38	AFG
		Microtus	Field vole (*Microtus agrestis*)	5	CH
			Common vole (*Microtus arvalis*)	101	CH
		Myodes	Bank vole (*Myodes glareolus*)	5, 1*	CZ, CH*
	Gliridae	*Eliomys*	Garden dormouse (*Eliomys quercinus*)	3	CH
Soricomorpha	Soricidae	*Crocidura*	White-toothed shrews (*Crocidura* sp.)	8	AFG
			Bicolored toothed-shrew (*Crocidura leucodon*)	20, 19*	CH, D*
			Greater white-toothed shrew (*Crocidura russula*)	1	CH
		Neomys	Mediterranean water shrew (*Neomys anomalus*)	4	CH
			Eurasian water shrew (*Neomys fodiens*)	2	CH
		Sorex	Long-tailed shrews (*Sorex* sp.)	83, 8*, 2**	CH, A*, D**
			Common shrew (*Sorex araneus*)	41, 8*	CH, D*
			Eurasian pygmy shrew (*Sorex minutus*)	14	CH
			Alpine Shrew (*Sorex alpinus*)	1	CH
	Erinaceinae	*Erinaceus*	European hedgehog (*Erinaceus europaeus*)	14	CH
	Talpidae	*Talpa*	European mole (*Talpa europaea*)	7	CH

[a] CH = Switzerland; AFG = Afghanistan; CZ = Czech Republic; D = Germany; A = Austria.

* Second *n* and corresponding origin.

** third *n* and corresponding origin.

Czech Republic, no detailed information was available besides the species and the origin. All samples besides those from Afghanistan were archived, formalin-fixed, and paraffin-embedded.

In summary 3,664 samples from 911 animals were investigated. Gastrointestinal tract samples ($n=688$) consisted of small intestine ($n=343$), large intestine ($n=330$), pancreas ($n=11$), esophagus ($n=3$), and stomach ($n=1$). Lymphatic tissue ($n=375$) included spleen ($n=368$) and lymph node ($n=7$). Testis ($n=5$), epididymis ($n=3$), mammary gland ($n=2$), and embryo ($n=2$) were included as reproductive tract samples ($n=12$). Species determination of animals from Afghanistan ($n=379$) was performed according to recently published protocols (Schlegel et al., 2012b). Species determination of animals positive or questionable positive for chlamydiae (Table 2) was performed by PCR (Schlegel et al., 2012b) and revealed a distinct sequence for a Mediterranean water shrew (*Neomys anomalus*) positive for *Waddlia* spp.

Sections of 30 μm of formalin-fixed, paraffin-embedded tissue samples were deparaffinized in xylene. After centrifugation at 13,500 × G for 5 min, the xylene was removed by repeated extraction in ethanol followed by a second centrifugation and the removal of residual ethanol (95%). The pellet was treated overnight with proteinase K (20 mg/mL, Roche Diagnostics GmbH, Mannheim, Germany) on a thermomixer (55 C, 550 rpm). The DNA was extracted using a commercial DNeasy Blood Tissue Kit and QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

The DNA concentration measurement was performed for each sample by measuring the absorbance at A260 nm and A260/280 absorbance ratio respectively on a spectrophotometer Nanodrop® 1000, Version 3.7.1. (Thermo Fisher Scientific, Wilmington, Delaware, USA). DNA content ranged from 1.1 to 821 ng/μL and showed an average value of 226 ng/μL. The optical density 260/280 ranged from 1.69 to 1.98 with an average of 1.87.

All samples were examined in duplicate on an ABI 7500 instrument (Applied Biosystems, Foster City, California, USA) using a 23S-rRNA gene-based *Chlamydiaceae* family-specific real-time PCR (Ehricht et al., 2006). Primers and probe (Microsynth, Balgach,

TABLE 2. Details of seven animals positive or questionable positive by real-time PCR for *Chlamydiaceae*, *Parachlamydia* spp., *Waddlia* spp. and *Chlamydiales*.[a]

Animal	Species	Origin	Sex	Age	Source	DNA concentration (ng/μm)
Vole	*Arvicola scherman*	Switzerland, Grisons	M	Adult	Small and large intestine	96.3
Shrew	*Crocidura leucodon*	Switzerland, Grisons	NA	Juvenile	Liver, lung, heart, kidney, spleen	71.7
Mouse	NA	Switzerland, Grisons	F	Adult	Feces	10.4
Shrew	NA	Switzerland, Zurich	F	Adult	Feces	68.7
Vole	*Microtus arvalis*	Switzerland, Grisons	M	Adult	Liver, lung, heart, kidney, spleen	153.7
Mouse	*Mus musculus*	Afghanistan	NA	NA	Liver	NA
Shrew	*Neomys anomalus*	Switzerland, Grisons	M	Adult	Liver, lung, heart, kidney	136.4

[a] Ø Ct value = mean threshold cycle value of at least two duplicates; secY qPCR = quantitative PCR targeting the secY gene; M = male; NA = not available; ND = not done; F = female.

Switzerland) were used as follows to amplify a 111-base pair (bp) product specific for the *Chlamydiaceae*: Ch23S-F (5′-CTGAAACCAGTAGCTTATAAGCGGT-3′), CH23S-R (5′-ACCTCGCCGTTTAACTTAACTCC-3′), and Ch23-p (FAM-CTCATCATGCAAAAGGCACGCCG-TAMRA). An internal amplification control was included consisting of primers EGFP-1-F (5′-GACCACTACCAGCAGAACAC-3′), EGFP-10-R (3′-CTTGTACAGCTCGTCCATGC-5′), and probe EGFP-HEX (HEX-AGCACCCAGTCCGCCCTGAGCA-BHQ1) to generate a 177-bp product. If the internal control was inhibited the sample was retested at a 1:10 dilution. To yield a final volume of 25 μL, 2.5 μL of DNA template were added to a mix of reagents containing 12.5 μL of 2× MasterMix-buffer (TaqMan® Fast Universal PCR Master Mix [Applied Biosystems]) and a final concentration of 500 nM of each primer and probe (Microsynth). The cycling profile included initial denaturation at 95 C for 10 min followed by 45 cycles of denaturation and amplification at 95 C for 15 sec and 60 C for 30 sec. An automatically calculated cycle threshold (Ct) value of <38 was considered positive. When both Ct values were <38 a sample was considered positive. If only one Ct value of <38 or a mean Ct value of >38 was obtained a sample was considered as questionable positive. Questionable positive samples were tested again in duplicate by the same method. Samples without a Ct value were interpreted as negative. *Chlamydia abortus* DNA was the positive control; the negative control was a reaction mix with water.

Samples with at least two Ct values were further examined by the species-specific 23S ArrayTube Microarray assay (Alere Chip Technologies GmbH, Jena, Germany) as described by Borel at al. (2008).

All *Chlamydiaceae* real-time PCR questionable positive samples were further investigated in duplicate by a 16S-rRNA-based *Chlamydiales*-specific real-time PCR, which should result in the generation of a 207–215 bp PCR product (variability in length depends on the species; Lienard et al., 2011). A final volume of 20 μL was obtained by adding a commercial mastermix (Bio-Rad, Reinach, Switzerland), 0.1 μM concentrations of each primer and probe (Eurogentec, Seraing, Belgium), molecular–biology-grade water (Sigma-Aldrich, Buchs, Switzerland), and 5 μL of DNA sample. The PCR run started with an initial denaturation (95 C, 3 min) followed by 50 cycles of denaturation, annealing, and extension (95 C, 15 sec; 67 C, 15 sec; 72 C, 15 sec).

Tissue samples (n=3,422) from 911 animals were examined with an ABI Prism 7500 instrument (Applied Biosystems) applying a modified version of a real-time PCR for the specific detection of *Parachlamydia* spp. as described by Casson et al. (2008a). This PCR assay should result in a 103-bp product of the 16S-rRNA gene specific for *Parachlamydia* spp. To yield a final volume of 25 μL, 0.5 μL of primer PacF (5′-CTCAACTCCAGAACAGCATTT-3′), 0.5 μl of primer PacR (5′-CTCAGCGTCAGGAATAAGC-3′), and 0.25

TABLE 2. Extended.

Real-time PCR for *Chlamydiaceae* (Ø Ct value)	16S-base *Chlamydiales* qPCR (Ø Ct value)	Real-time PCR for *Parachlamydia* (Ø Ct value)	*Parachlamydia* secY qPCR (Ø Ct Value)	Real-time PCR for *Waddlia* (Ø Ct value)	*Waddlia* secY qPCR (Ø Ct value)
Questionable positive (40.1)	Negative	Negative	Negative	Negative	Negative
Negative	ND	Positive (38.8)	Negative	Negative	ND
Negative	ND	Positive (39.3)	Negative	Negative	ND
Negative	ND	Positive (40.5)	Negative	Negative	ND
Negative	ND	Positive (41.6)	Negative	Negative	ND
Negative	ND	Positive (42.3)	Negative	Negative	ND
Negative	ND	Negative	ND	Positive (38.9)	Positive (39.0)

probe PacS (5′-tetrachloro-6-carboxyfluoresceinTTCCACATGTAGCGGTGAAATGCGTAGATATG-Black Hole Quencher 1–3′) (Applied Biosystems), each with a final concentration of 10 μM, were added to a reaction mix of 2.5 μL DNA sample and 12.5 μL iTaqSupermix® (Bio-Rad). The cycling conditions were 3 min at 95 C for initial denaturation, followed by 45 cycles of denaturation, and amplification for 15 sec at 95 C and 1 min at 60 C. Samples were tested at least in duplicate and were considered negative if no amplification was observed during all 45 cycles (Blumer et al., 2011).

For *Waddlia*-specific DNA amplification, the cycling conditions were the same as for *Parachlamydia*. Primers WadF, WadR, and probe WadS were used as described by Goy et al. (2009) to generate a 101-bp product within the 16S-rRNA gene specific for *Waddlia* spp. In total, 746 samples were tested using this PCR assay. A plasmid containing a part of the 16S gene sequence of *Parachlamydia* spp. or *Waddlia* spp. was used as a positive control, respectively. As negative control, a reaction mixture with water (H_20) was amplified. Samples were tested at least in duplicate and considered negative if no amplification was observed during all 45 cycles (Blumer et al., 2011).

All samples positive or questionable positive with the pathogen-specific PCR targeting the 16S-rRNA gene were confirmed by PCR targeting the secY encoding gene of *P. acanthamoebae* and *W. chondrophila*, respectively. To yield a final volume of 20 μL, 0.4 μL (200 nM) of primer secY_Parachlam_F2 (5′-GCTCTTTGGCCATCTCTACAGCGT-3′), 0.4 μL (200 nM) of primer secY_Parachlam_R2 (5′-CCAGCGACGATACCTGGCTTTGA-3′), and 0.2 μL (100 nM) of probe secY_Parachlam_S2 (5′-FAM-ACGCGAAAATGGGAAGCGGCAAAGCG-BHQ1-3′) were added to a reaction mix of 5 μL DNA sample and 10 μL of iTaqSupermix with ROX (Bio-Rad). In the same manner, primers and probe for *W. chondrophila* were used as follows: secY_Wad_F1 (5′-CGCCAAGGGCGTCCAACTCA-3′), secY_Wad_R1 (5′-AGCAAAGCCGTACCGCCAAAGA-3′), and secY_Wad_S1 (5′-FAM-ACTCTCATCGGCGCGGTTTTCCTTGCT-BHQ1-3′). Seven tissue samples were examined in duplicate with a StepOnePlus™ (Life Technologies, Carlsbad, California, USA) instrument. The cycling conditions were the same as for the 16S-rRNA PCR for *Parachlamydia* spp. or *Waddlia* spp., respectively. Both species-specific secY PCR assays amplify a DNA region of about 100 bp.

RESULTS

Of 3,664 samples from 911 animals, one organ sample consisting of the small and large intestine from one vole (0.11%) was questionable positive by real-time PCR for *Chlamydiaceae* with a mean threshold cycle (Ct) value of 40.1 (Table 2). This result could not be confirmed by the *Chlamydiales* 16S-rRNA PCR, and species identification by ArrayTube Microarray was not conclusive.

Thirteen tissue samples (three liver and two each lung, heart, kidney, spleen, and feces) from five animals (0.55%) were positive by real-time PCR for *Parachlamydia* spp. with mean Ct values between 38.8 and 42.6. However, results of these 13 samples could not be confirmed by the *Parachlamydia* secY qPCR.

Samples from 746 animals were available to test for *Waddlia*. Therefrom, the organ pool consisting of liver, lung, heart, and kidney from one animal (0.13%) had a mean Ct value of 38.9 by real-time PCR for *Waddlia* spp. This was confirmed by *Waddlia* secY qPCR (Ct value 39.0).

DISCUSSION

Samples of 679 rodents and 232 insectivores including shrews, hedgehogs, and moles were investigated (Table 1). To the authors' knowledge, the current study is the first large-scale screening for *Chlamydiaceae*, and in particular for *C. muridarum*, in wild rodent and insectivore species in European countries and Afghanistan. DNA of *C. muridarum* was not detected. Previous studies in laboratory mice indicate that a low-level infection without overt disease might be common (Karr, 1943). However, a transient infection of young animals could also be possible (Fox et al., 2006). In our study transient infection might not have been detected because samples were only available at a particular time. Because all 688 organ samples of the gastrointestinal tract were negative by *Chlamydiaceae*-specific real-time PCR, wild small mammals are probably not carriers of intestinal chlamydiae or the prevalence of infection is very low. Despite this, it is also possible that wild small mammals do not normally harbor *C. muridarum*. However, a recent preliminary study has detected chlamydial infection in a New World cricetide rodent, i.e., *Peromyscus* spp. (Ramsey et al., unpubl. data).

For an adult water vole captured in the canton of Grisons, where *C. abortus* is endemic in small ruminants (Borel et al., 2004), a questionable result was obtained in the *Chlamydiaceae* real-time PCR but was negative for *C. abortus*. However, the absence of *C. abortus* in our study is in line with recent surveys in wild ruminants from Switzerland including ibex (*Capra ibex*; 4/412 positive), red deer (*Cervus elaphus*) and roe deer (*Capreolus capreolus*; all 163 negative), and chamois (*Rupicapra rupicapra*; 1/79 positive) tested by the same methods (Holzwarth et al., 2011a, b; Regenscheit et al., 2012).

All investigated individuals were also negative for *Chlamydiaceae* other than *C. muridarum* and *C. abortus*. These findings indicate that neither wild ruminant species nor free-ranging rodent and insectivore species act as significant reservoir or transport hosts for *C. abortus*, at least not in the examined geographic regions. Previous studies in the literature often rely on serologic methods of low sensitivity and specificity such as complement fixation (Wilson et al., 2009). Complement fixation is cross-reactive with other chlamydial species, which may have led to false-positive results (Griffiths et al., 1996). However, in our study sensitive and specific direct TaqMan real-time PCR was carried out on formalin-fixed, paraffin-embedded organ and fecal samples.

We found 13 samples, including internal organs such as liver, lung, heart, kidney, spleen, and feces, from five 911 (0.55%) positive by real-time PCR for *Parachlamydia* spp. but negative by the *Parachlamydia* secY qPCR. Despite the restricted availability of GenBank-deposited *Chlamydiales* secY sequences, the alignment of several *Chlamydiales* secY sequences indicates that *P. acanthamoebae* secY quantitative PCR is specific at the species level (Fig. 1). Only one mismatch was detected between the strains *Parachlamydia acanthamoebae* Hall coccus and UV-7, which should not prevent PCR amplification. The secY alignment suggests that the quantitative secY PCR is more specific than the 16S-rRNA PCR due to reduced sequence conservation of secY

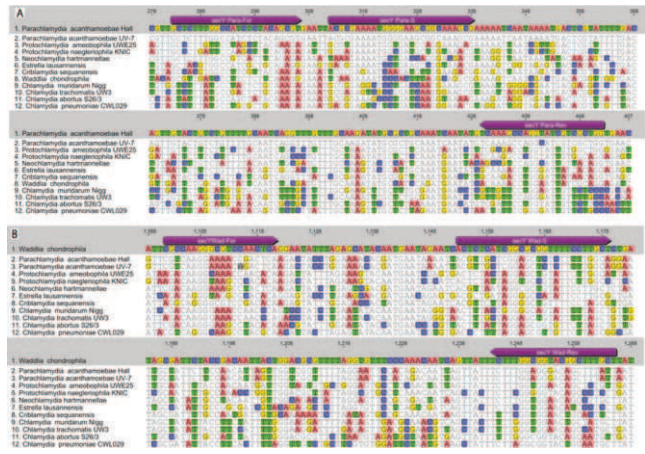

FIGURE 1. Alignment of the primers and probes used for quantitative real-time PCRs targeting the secY genes of *Parachlamydia* and *Waddlia* spp. (A) Nucleotide sequence alignment of Chlamydiales secY gene regions targeted by the secY quantitative real-time PCRs of *Parachlamydia acanthamoebae* strain Hall coccus. Primers (secY Para-For and secY Para-Rev) and probe (secY Para-S) of the quantitative real-time PCR are indicated by purple arrows. Highlighted bases represent mismatches to the reference sequence of *P. acanthamoebae* strain Hall coccus. (B) Nucleotide sequence alignment of Chlamydiales secY gene regions targeted by the secY quantitative real-time PCRs of *Waddlia chondrophila*. Primers (secY Wad-For and secY Wad-Rev) and probe (secY Wad-S) of the quantitative real-time PCR are indicated by purple arrows. Highlighted bases represent mismatches to the reference sequence of *W. chondrophila.*

genes compared to 16S-rRNA genes. Thus, these five animals detected as positive by the *Parachlamydia* spp. 16S-rRNA real-time PCR but negative by the *P. acanthamoebae* secY PCR may be infected by a species related to *P. acanthamoebae* but that exhibits enough difference in the secY target to prevent PCR amplification. Under experimental conditions, parachlamydial infection in laboratory mice was demonstrated by intratracheal inoculation of *P. acanthamoebae*, producing an acute purulent to interstitial pneumonia with a mortality rate of 50% within 5 days (Casson et al., 2008b). Pathogenicity in mice was also confirmed by another in vivo study of the role of toll-like receptors in the sensing of *P. acanthamoebae* (Roger et al., 2010). Despite this, the role of wild small mammals as either a reservoir or carrier host of *Parachlamydia* spp. remains unclear because only results with high Ct values (38.8 to 42.3) were obtained.

A pooled organ sample (liver, lung, heart, and kidney) from one animal (0.13%) was positive by real-time PCR for *Waddlia* spp., and this result was confirmed by the *Waddlia* secY qPCR. This positive result with two PCR methods targeting different DNA regions confirms that the shrew may indeed have been infected by *Waddlia* spp. To our knowledge this is the first detection of *Waddlia* in internal organs of a wild Mediterranean water shrew from Grisons, Switzerland. Scarce data are available on the prevalence of *Waddlia* spp. and its impact in wildlife. Recently a novel *Waddlia* species was isolated from urine samples of fruit bats (*Eonycteris spelaea*) in Malaysia (Chua et al., 2005). *Waddlia chondrophila* is considered as a *Chlamydia*-like organism associated with abortion in ruminants (Henning et al., 2002; Dilbeck-Robertson et al., 2003) and humans (Baud et al., 2011). In 2007 Baud et al. showed that seven of 200 women with recurrent miscarriages and two of 97 women with antibody to *Waddlia* spp. had prior contact with rodents.

We conclude that *Chlamydiaceae* are absent or occur very rarely in free-living

rodents and insectivores, at least in the populations that we screened. Similarly, these small wild mammals may be only sporadically infected with *Parachlamydia* spp. and *Waddlia* spp. However, further studies in other geographic areas investigating other small wild rodent and insectivore species are needed to shed light on the potential pathogenicity, distribution, and transmission of chlamydiae in wildlife populations of underestimated importance.

ACKNOWLEDGMENTS

We thank Robert V. Schoborg, Department of Microbiology, Quillen College of Medicine, East Tennessee State University, Johnson City, Tennessee for help with manuscript preparation. We are grateful to Jürg Paul Müller of the Natural History Museum of the Grisons for support in species identification of small mammals. Many thanks to F. Burach, F. Ehrensperger, M. Puorger, S. Ruegg and T. Sydler for assistance in sample collection. We are grateful to the laboratory technical staff (in particular Carmen Kaiser) of the Institute of Veterinary Pathology, Vetsuisse Faculty, University of Zurich for technical help. Many thanks to Sébastian Aeby from the Microbiology Institute of the University of Lausanne for performing parts of the PCR examination. We thank P. Deplazes and D. Hegglin for collaboration. The development of the secY PCR in G. Greub's group was supported by a SNSF grant 310030-130466.

SUPPLEMENTARY MATERIAL

Supplementary material for this article is online at http://doi:10.7589/xxxx-xx-xxx.

LITERATURE CITED

Baud D, Thomas V, Arafa A, Regan L, Greub G. 2007. *Waddlia chondrophila*, a potential agent of human fetal death. *Emerg Infect Dis* 13:1239–1243.

Baud D, Goy G, Osterheld MC, Borel N, Vial Y, Pospischil A, Greub G. 2011. *Waddlia chondrophila*: From bovine abortion to human miscarriage. *Clin Infect Dis* 52:1469–1470.

Blumer S, Greub G, Waldvogel A, Hässig M, Thoma R, Tschuor A, Pospischil A, Borel N. 2011. *Waddlia*, *Parachlamydia* and *Chlamydiaceae* in bovine abortion. *Vet Microbiol* 152:385–393.

Borel N, Doherr MG, Vretou E, Psarrou E, Thoma R, Pospischil A. 2004. Seroprevalences for ovine enzootic abortion in Switzerland. *Prev Vet Med* 65:205–216.

Borel N, Kempf E, Hotzel H, Schubert E, Torgerson P, Slickers P, Ehricht R, Tasara T, Pospischil A, Sachse K. 2008. Direct identification of chlamydiae from clinical samples using a DNA microarray assay—A validation study. *Mol Cell Probes* 22:55–64.

Caro MR, Buendìa AJ, Del Rio L, Ortega N, Gallego MC, Cuello F, Navarro JA, Sanchez J, Salinas J. 2009. *Chlamydophila abortus* infection in the mouse: A useful model of the ovine disease. *Vet Microbiol* 135:103–111.

Casson N, Posfay-Barbe KM, Gervaix A, Greub G. 2008a. New diagnostic real-time PCR for specific detection of *Parachlamydia acanthamoebae* DNA in clinical samples. *J Clin Microbiol* 46:1491–1493.

Casson N, Entenza JM, Borel N, Pospischil A, Greub G. 2008b. Murine model of pneumonia caused by *Parachlamydia acanthamoebae*. *Microb Pathog* 45:234–236.

Chua PKB, Corkill JE, Hooi PS, Choon Cheng S, Winstanley C, Hart CA. 2005. Isolation of *Waddlia malaysiensis*, a novel intracellular bacterium, from fruit bat (*Eonycteris spelaea*). *Emerg Infect Dis* 11:271–277.

Codony F, Fittipaldi M, Lòpez E, Moratò J, Agutsì G. 2012. Well water as a possible source of *Waddlia chondrophila* infections. *Microbes Environ* 27:529–532.

Deuchande R, Gidlow J, Caldow G, Baily J, Longbottom D, Wheelhouse N, Borel N, Greub G. 2010. *Parachlamydia* involvement in bovine abortions in a beef herd in Scotland. *Vet Rec* 166:598–599.

Dilbeck-Robertson P, McAllister MM, Bradway D, Evermann JF. 2003. Results of a new serological test suggest an association of *Waddlia chondrophila* with bovine abortion. *J Vet Diagn Invest* 15:468–469.

Dochez AR, Mills KC, Mulliken B. 1937. A virus disease of Swiss mice transmissible by intranasal inoculation. *Proc Soc Biol Med* 36:683–686.

Ehricht R, Slickers P, Goellner S, Hotzel H, Sachse K. 2006. Optimized DNA microarray assay allows detection and genotyping of single PCR-amplifiable target copies. *Mol Cell Probes* 20:60–63.

Everett KDE, Bush RM, Andersen AA. 1999. Emended description of the order Chlamydiales, proposal of *Parachlamydiaceae* fam. nov. and *Simkaniaceae* fam. nov., each containing one monotypic genus, revised taxonomy of the family *Chlamydiaceae*, including a new genus and five new species, and standards for the identification of organisms. *Int J Syst Bacteriol* 49:415–440.

Goy G, Croxatto A, Posfay-Barbe KM, Gervaix A, Greub G. 2009. Development of a real-time PCR for the specific detection of *Waddlia chondrophila* in clinical samples. *Eur J Clin Microbiol Infect Dis* 28:1483–1486.

Greub G, Raoult D. 2002. *Parachlamydiaceae*: Potential emerging pathogens. *Emerg Infect Dis* 8:625–630.

Griffiths PC, Plater JM, Horigan MW, Rose MPM, Venables C, Dawson M. 1996. Serological diagnosis of ovine enzootic abortion by comparative inclusion immunofluorescence assay, recombinant lipopolysaccharide enzyme-linked immunosorbent assay. *J Clin Microbiol* 34:1512–1518.

Henning K, Schares G, Granzow H, Polster U, Hartmann M, Hotzel H, Sachse K, Peters M, Rauser M. 2002. *Neospora caninum* and *Waddlia chondrophila* strain 2032/99 in a septic stillborn calf. *Vet Microbiol* 85:285–292.

Hilbe M, Herrsche R, Kolodziejek J, Nowotny N, Zlinszky K, Ehrensperger F. 2006. Shrews as reservoir hosts of Borna disease virus. *Emerg Infect Dis* 12:675–677.

Holzwarth N, Pospischil A, Marreros N, Ryser-Degiorgis MP, Mavrot F, Frey J, Thoma R, Borel N. 2011a. Alpine ibex (*Capra i. ibex*) is not a reservoir for chlamydial infections of domestic ruminants and humans. *Eur J Wildl Res* 57:233–240.

Holzwarth N, Pospischil A, Mavrot F, Vilei EM, Hilbe M, Zlinsky K, Regenscheit N, Pewsner M, Thoma R, Borel N. 2011b. Occurrence of *Chlamydiaceae*, *Mycoplasma conjunctivae*, and pestiviruses in alpine chamois *(Rupicapra r. rupicapra)* of Grisons, Switzerland. *J Vet Diagn Infest* 23:333–337s.

Karr HV. 1943. Study of a latent pneumotropic virus of mice. *J. Infect. Dis* 72:108–116.

Laitinen K, Alakärppä H, Laurila A, Leinonen M. 1997. Animal models for *Chlamydia pneumonia* infection. *Scand J Infect Dis*, Suppl 104: 15–17.

Lienard J, Croxatto A, Aeby S, Jaton K, Posfay-Barbe K, Gervaix A, Greub G. 2011. Development of a new *Chlamydiales*-specific real-time PCR and its application to respiratory clinical samples. *J Clin Microbiol* 49:2637–2642.

Longbottom D, Coulter LJ. 2003. Animal chlamydioses and zoonotic implications. *J Comp Path* 128:217–244.

Lutz-Wohlgroth L, Becker A, Brugnera E, Huat ZL, Zimmermann D, Grimm F, Haessig M, Greub G, Kaps S, Spiess B, et al. 2006. *Chlamydiales* in guinea-pigs and their zoonotic potential. *J Vet Med A* 53:185–193.

Nigg C. 1942. An unidentified virus which produces pneumonia and systemic infection in mice. *Science* 95:49–50.

Nigg C, Eaton MD. 1944. Isolation from normal mice of a pneumotropic virus which forms elementary bodies. *J Exper Med* 79:497–510.

Puorger ME, Hilbe M, Muller JP, Kolodziejek J, Nowotny N, Zlinszky K, Ehrensperger F. 2010. Distribution of Borna disease virus antigen and RNA in tissues of naturally infected bicolored white-toothed shrews, *Crocidura leucodon*, supporting their role as reservoir host species. *Vet Pathol* 47:236–244.

Rank RG. 2006. Chlamydial diseases. In: *The mouse in biomedical research*, Fox JG, Barthold SW, Davisson MT, Newcomer CE, Quimby FW, Smith AL, editors. Elsevier Academic Press, San Diego, California, pp. 325–348.

Regenscheit N, Holzwarth N, Greub G, Aeby S, Pospischil A, Borel N. 2012. Deer as a potential wildlife reservoir for *Parachlamydia* species. *Vet J* 193:589–592.

Richter M, Matheis F, Gönczi E, Aeby S, Spiess B, Greub G. 2010. *Parachlamydia acanthamoebae* in domestic cats with and without corneal disease. *Vet Ophthalmol* 13:235–237.

Roger T, Casson N, Croxatto A, Entenza JM, Pusztaszeri M, Akira S, Knaub Reymond M, Le Roy D, Calandra T, Greub G. 2010. Role of MyD88 and Toll-Like Receptors 2 and 4 in the sensing of *Parachlamydia acanthamoebae*. *Infect Immun* 78:5195–5201.

Ruhl S, Casson N, Kaiser C, Thoma R, Pospischil A, Greub G, Borel N. 2009. Evidence for *Parachlamydia* in bovine abortion. *Vet Microbiol* 135:169–174.

Schlegel M, Baumann K, Breithaupt A, Binder A, Schotte U, Ruhl S, Krohmann C, Essbauer S, Fragoulidis D, Kasser P, et al. 2012a. What about the role of rodents as vectors for zoonotic pathogens in mission areas of the Bundeswehr? *Wehrmedizinische Monatszeitschrift* xx:8–9.

Schlegel M, Sheikh Ali H, Stieger N, Groschup MH, Wolf R, Ulrich RG. 2012b. Molecular identification of small mammal species using novel *Cytochrome b* gene-derived degenerated primers. *Biochem Genet* 50:440–447.

Stills H, Fox JG, Plaster BJ, Dewhirst FE. 1991. A "new" *Chlamydia* sp. strain SFPD isolated from transmissible proliferative ileitis in hamsters. *Microb Ecol Health D* 4, v. S99 (special).

Storz J, Page AL. 1971. Taxonomy of the *Chlamydiae*: Reasons for classifying organisms of the genus *Chlamydia*, family *Chlamydiaceae*, in a separate order, *Chlamydiales* ord. nov. *Int J Syst Bacteriol* 21:332–334.

Wheelhouse N, Sait M, Gidlow J, Deuchande R, Borel N, Baily J, Caldow G, Longbottom D. 2011. Molecular detection of *Chlamydia*-like organisms in cattle drinking water. *Vet Microbiol* 152:196–199.

Wilson K, Livingstone M, Longbottom D. 2009. Comparative evaluation of eight serological assays for diagnosing *Chlamydophila abortus* infection in sheep. *Vet Microbiol* 135:38–45.

Zhang Y, Fox JG, Ho Y, Zhang L, Stills HF, Smith TF. 1993. Comparison of the major outer-membrane protein (MOMP) gene of mouse pneumonitis (MoPn) and hamster SFPD strains of *Chlamydia trachomatis* with other *Chlamydia* strains. *Mol Biol Evol* 10:1327–1342.

Submitted for publication 2 August 2013.
Accepted 5 October 2013.